

PROCESS FOR EXTRACTING PURE FRACTIONS OF LACTOPEROXIDASE AND LACTOFERRIN FROM MILK SERUM.

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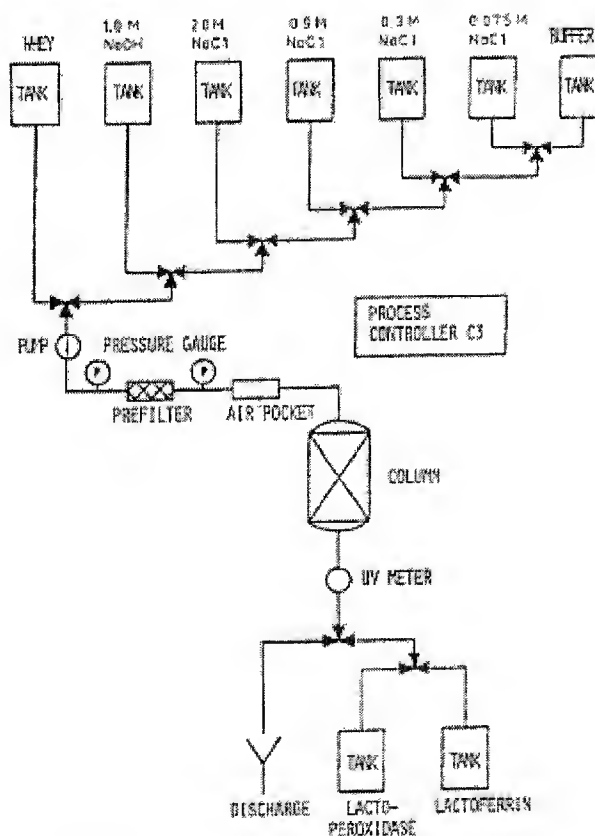
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Abstract not available for EP0390821

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PCT No. PCT/SE88/00643 Sec. 371 Date May 24, 1990 Sec. 102(e) Date May 24, 1990 PCT Filed Nov. 25, 1988 PCT Pub. No. WO89/04608 PCT Pub. Date Jun. 1, 1989. A process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum is described. The milk serum is microfiltered and passed through a strong cation exchanger at a high rate of flow for selective adsorption of lactoperoxidase and lactoferrin, and then the lactoperoxidase and lactoferrin are eluted successively and selectively with saline solutions having different concentrations.



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<p>(51) International Patent Classification⁴ : A23J 1/20, C07K 3/28 C12N 9/08, A23C 9/146</p>	<p>A1</p>	<p>(11) International Publication Number: WO 89/ 04608 (43) International Publication Date: 1 June 1989 (01.06.89)</p>
<p>(21) International Application Number: PCT/SE88/00643 (22) International Filing Date: 25 November 1988 (25.11.88) (31) Priority Application Number: 8704719-7 (32) Priority Date: 27 November 1987 (27.11.87) (33) Priority Country: SE (71) Applicant (for all designated States except US): SVENSKA MEJERIERNAS RIKSFÖRENINGEN EKONOMI-AKTIEBOLAG [SE/SE]; Box 24, S-101 20 Stockholm (SE). (72) Inventor; and (75) Inventor/Applicant (for US only) : BURLING, Hans [SE/SE]; Hövdängavägen 15, S-223 75 Lund (SE). (74) Agent: AWAPATENT AB; Box 5117, S-200 71 Malmö (SE).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US. Published With international search report. With amended claims. In English translation (filed in Swedish).</p>
<p>(54) Title: PROCESS FOR EXTRACTING PURE FRACTIONS OF LACTOPEROXIDASE AND LACTOFERRIN FROM MILK SERUM</p> <p>(57) Abstract</p> <p>A process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum is described. The milk serum is microfiltered and passed through a strong cation exchanger at a high rate of flow for selective adsorption of lactoperoxidase and lactoferrin, and then the lactoperoxidase and lactoferrin are eluted successively and selectively with saline solutions having different concentrations.</p>		

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PROCESS FOR EXTRACTING PURE FRACTIONS OF
LACTOPEROXIDASE AND LACTOFERRIN FROM MILK SERUM

The present invention relates to a process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum. By milk serum is meant both skim milk and whey.

5 In cheese-making, a large amount of whey is obtained as a by-product. Whey has a dry solids content of about 6%, which is composed approximately as follows:

	% by weight
10 Lactose	4.6
Protein	0.6 thereof
Lactoperoxidase	0.0020
Lactoferrin	0.0030
Fat	0.05 (after separation)
15 Salts	<u>0.7</u>
Dry solids content about	6.0

The protein fraction which constitutes about 10-12% of the dry solids content is composed of a number of different protein components. The biggest
 20 are β -lactoglobulin, α -lactalbumin and bovine serum-albumin. Also a number of bioactive components belong to the protein fraction, for example immunoglobulins, lactoperoxidase, lactoferrin and lysozyme.

25 Both lactoperoxidase and lactoferrin have anti-microbial properties. There is a great interest in extracting natural antimicrobial substances to be used in new contexts in food technology and in the chemico-technical and medical fields.

30 There are low contents of these substances in skim milk and whey (also in the original milk). Lactoperoxidase and lactoferrin are present in contents of 15-50 mg/litre, depending on the lactation state of the cow. Large quantities of whey (milk) must thus

be filtered to facilitate extration of kilogram amounts of these bioactive components.

The process engineering conditions for isolating lactoperoxidase and lactoferrin, respectively, from milk/whey are based on the fact that the isoelectric point (pI) for these two proteins is about 9.5, while the main part of the whey proteins have isoelectric points around 5.1-5.4 and the casein at about 4.6. A fundamentally suitable process for separation of lactoperoxidase and lactoferrin is therefore to contact the milk/whey with a cation exchanger at a pH of < 6 for selective adsorption, and use is here made of the positive net charge of lactoperoxidase and lactoferrin, which distinguishes from that of other milk proteins which have a negative charge at this pH.

The traditional way of isolating lactoperoxidase and lactoferrin in small amounts for the purpose of research is to use the precipitation technique and ion exchange chromatography, frequently combined with gel filtration, see Morrison, M., Hamilton, H-B., Stotz, E., J. Biol. Chem. 228:767 (1957); and Morrison, M., Hultquist, P-E., J. Biol. Chem. 238-2847 (1963). These methods are not suited for preparing large amounts of the bioactive components at issue in an economically defensible manner.

U.S. patent specification 4,436,658 (Pevrosuset) discloses adsorption of lactoferrin from casein-free milk serum (whey) by means of a silica column. The pH of the milk serum is adjusted to 7.7-8.2 before adsorption on the column. Immunoglobulins, lactoferrin and lactoperoxidase adhere to the column. After the adsorption phase, elution with a diluted saline solution at a pH of < 4 takes place. No selective elution of the adsorbed proteins is obtained, particularly not regarding lactoperoxidase. A column holding about 5 g of silica compound can treat 1 litre of whey. This prior art process must be regarded as unsuitable for

application on an industrial scale.

Zagulski et al. in *Prace in Materialy Zootechniczne* 20, (1979), p. 87-103 describes a batchwise method of obtaining lactoferrin, in which use is made of a weak cation exchanger which is mixed with milk. After equilibration, the ion exchanger is applied to a column for elution of the adsorbed proteins with a saline solution. The method thus is based on a batchwise process, and a further purification must be carried out in a second ion exchange step to obtain a high purity of the lactoferrin.

A similar process is described in BE patent specification 901,672 (J.P. Prieels and R. Peipper, Oleofina S.A.). Here, use is made of an ion exchanger based on calcium alginate, in which the ion exchange functionality has been obtained by admixture of oxides of zirconium, titanium, quartz or aluminium. The milk/whey is contacted with the ion exchanger in a packed column or by mixing in a tank, whereby proteins having an isoelectric point above 7.5 are adsorbed. After equilibration, the gel is separated mechanically and supplied to a means for washing and eluting with a calcium chloride solution. All fluids contacting the calcium alginate gel must contain at least 0.1% CaCl_2 to prevent the gel from being dissolved. No fractionating of lactoperoxidase and lactoferrin is obtained in the elution, but the fractionating must be carried out in a separate purification step.

As a reason for not working with a commercially established ion exchange technique in a column process, the above-mentioned BE patent specification mentions the unsurmountable difficulties of clogging of the ion exchanger caused by the occurrence of particles of globular fat and protein aggregate in the medium.

GB patent specification 2,179,947 discloses a process for the extraction of lactotransferrin from milk. The process is carried out such that the whey

is subjected to ultrafiltration, whereby the protein content of the whey (including the lactotransferrin) is concentrated about 5 times, whereupon its pH and ionic strength are adjusted. The milk serum thus treated
5 is passed at a very low rate (about 0.03 bed volumes per minute) through an ion exchange column, preferably a weak cation exchanger. The column is eluted, still at a low rate, with a solution having an ionic strength gradient which increases up to 0.4 M, when the lacto-
10 transferrin is eluted. This is a small-scale process which is not suitable for industrial preparation of lactoferrin. The use of a weak cation exchanger results in a poor capacity. Whey in an amount of 100 bed volumes, converted into a natural dry solids content, can pass
15 through the ion exchange column between each elution. The problem of clogging of the ion exchange filter caused by fat and protein particles has not been solved by this prior art process.

The following requirements can be placed on an
20 industrially applicable process for economic recovery of lactoperoxidase and lactoferrin from whey/skim milk:

1) High selective capacity of the adsorption mass.

Since the contents of lactoperoxidase/lactoferrin are low in milk serum, the volumes of milk serum
25 which can be treated in one elution, must be large.

2) High rate of flow in the adsorption phase. (Normal chromatographic processes usually work at low rates, 0.01-0.10 bed volumes per minute. The reason is
30 that owing to the small particle size, the bed usually gives high pressure drops, and that the reaction kinetics for the adsorption process frequently require a high rate of flow.

3) The process must be hygienic, which means that the adsorption mass must stand at least a lye
35 treatment at pH 13-14.

The object of the present invention is to achieve a process that satisfies the above-mentioned requirements

for extraction of pure fractions of lactoperoxidase and lactoferrin from milk serum (whey) on a large scale and at a low cost.

The present invention relates to a process for
5 extracting pure fractions of lactoperoxidase and lactoferrin from milk serum, said process being characterised by microfiltering the milk serum, passing it through a strong cation exchanger at a high rate of flow for selective adsorption of lactoperoxidase and lactoferrin,
10 and successively eluting the lactoperoxidase and the lactoferrin selectively with saline solutions having different concentrations.

According to the invention, a process is provided for preparing pure fractions of two different serum
15 proteins in a single ion exchange step. This has not previously been achieved on an industrial scale. Prior art methods for extracting these proteins on an industrial scale have required two or three purification steps.

The above-mentioned problems of clogging of the
20 ion exchanger, which is caused by the occurrence of particles, such as globular fat and protein aggregates, in the serum or whey, are solved according the invention in that the milk serum (whey) is microfiltered, for example in a so-called cross-flow process, before
25 contacting the ion exchange bed. By choosing a suitable pore size of the microfilter, fat and protein aggregate particles which cause clogging, can be eliminated. A suitable microfilter has a pore diameter of 0.10-2 μm , preferably 0.4-1.5 μm .

30 As the starting material for the process according to the invention, milk serum (whey) is used, i.e. milk freed from fat and casein. The milk serum is first treated by microfiltration for removal of residues of fat and protein aggregate particles, preferably
35 in a so-called cross-flow process. The microfiltered milk serum is then passed at a high rate (about 1-1.5 bed volumes per minute) through a column packed with

a strong cation exchanger which selectively adsorbs lactoperoxidase and lactoferrin. This cation exchanger has excellent rate and adsorption kinetic properties and a capacity of about 1000 bed volumes of milk serum.

5 This means that about 1000 bed volumes of milk serum can pass before the lactoperoxidase which has the weakest bond, breaks through, i.e. the ion exchange mass is saturated with these proteins. Merely a slight increase of the pressure drop occurs between the be-
10 ginning and the end of the adsorption phase.

The elution of the ion exchange mass is started by washing the milk serum out of the column with a buffer, preferably a phosphate buffer at the pH of the milk serum, 6.5. Subsequently, impurities, if
15 any, are eluted with a buffer solution containing a weak saline solution, preferably of an inorganic alkali, alkaline earth or ammonium salt, for example 0.075 M NaCl.

After this preparatory elution, the desired proteins
20 are selectively eluted with buffer solutions containing saline solutions selected from the above-mentioned salts, at different concentrations. Thus, the elution of lactoperoxidase is performed at a salt concentration in the range of 0.10-0.4 M, and of lactoferrin at
25 a salt concentration within 0.5-2 M.

After this treatment, the proteins concerned have been concentrated about 500 times.

The pure protein fractions are collected, and then a further concentration is preferably effected by
30 ultrafiltration followed by desalination and freeze-drying so as to obtain a commercial product consisting of about 90% pure protein fractions.

For the production of 1 kg lactoperoxidase and 1 kg lactoferrin, about 65 and, respectively, 45 m³ of
35 whey are required. The purity of the extracted components exceeds 90%. This is obtained by a suitable choice of ion exchanger and a careful choice of

adsorption and elution conditions in which the pH and the salt concentrations are important parameters.

The invention will now be described in detail by means of the Example below and the accompanying drawings.

5 Fig. 1 is a schematic view of a preferred embodiment of the process according to the invention;

Fig. 2 illustrates the UV absorption spectrum when eluting lactoperoxidase and lactoferrin from an ion exchange column; and

10 Fig. 3 are chromatograms showing the purity of lactoperoxidase and lactoferrin after the fractionating according to the invention has been carried out.

Example

100 litres of pasteurised and sludge-centrifuged sweet whey at pH 6.5 were microfiltered in a cross-flow process at 50°C. By the microfiltration, remaining residues of globular fat were removed together with occurring protein aggregates. The pore size of the microfilter was 1.4 µm.

20 After cooling, the whey was passed through an ion exchange column packed with 80 ml of a specially treated strong cation exchanger (S-Sepharose, fast flow, Pharmacia) on an agarose basis. The height of the bed was about 4.1 cm, and the rate through the column was 100 ml/minute, corresponding to a rate of 1.25 bed volumes per minute. The pressure drop before the column at the beginning of the run was 0.26 bar. 15 h later, the rate was still 100 ml/minute at a pressure drop of 0.28 bar. The lactoperoxidase break-through occurred when about 80-90 litres of whey had been passed through the column, i.e. about 1000 bed volumes.

30 Subsequently, the flow of whey was interrupted, and the eluting phase was started by washing the whey out of the column with a phosphate buffer, 0.01 M KH_2PO_4 , pH 6.5, followed by elution of impurities from the ion exchanger with a phosphate buffer containing 0.075 M NaCl (Fig. 1). The lactoperoxidase

was eluted with a phosphate buffer containing 0.3 M NaCl, and then the lactoferrin was eluted with a phosphate buffer containing 0.9 M NaCl (see Fig. 2).

- 5 After the fractions had been collected, they were desalted by gel filtration in a Sephadex column and were finally freeze-dried.

The ion exchange column was cleaned by washing first with 2.0 M NaCl and then with 1.0 M NaOH, whereupon the column was ready for the next run.

- 10 - Yield of lactoperoxidase after ion exchange: 96.5%.
- Purity of the collected fraction after elution:
 $A_{412}/A_{280} = 0.84^*$
- Total yield in the process after
freeze-drying, calculated as activity: 90%
15 - Purity of the freeze-dried
preparation: $A_{412}/A_{280} = 0.87^*$
*0.92 is the maximum quota for 100% purity.

The corresponding yield and purity were obtained for the lactoferrin (see Fig. 3).

CLAIMS

1. A process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum, characterised by microfiltering the milk serum, passing it through a strong cation exchanger at a high rate of flow for selective adsorption of lactoperoxidase and lactoferrin, and then successively and selectively eluting the lactoperoxidase and the lactoferrin with saline solutions having different concentrations.
2. The process as claimed in claim 1, characterised in that the lactoperoxidase is selectively eluted with a saline solution having a concentration of 0.10-0.4 M at a pH of about 6.5.
3. The process as claimed in claim 1 or 2, characterised in that the lactoferrin is selectively eluted with a saline solution having a concentration of 0.5-2 M.
4. The process as claimed in one or more of the preceding claims, characterised in that before the elution of lactoperoxidase, the cation exchanger is eluted with a saline solution having a concentration of 0.01-0.15 M, preferably a solution of an inorganic alkali, alkaline earth or ammonium salt.
5. The process as claimed in one or more of the preceding claims, characterised in that the pH of the milk serum is adjusted to 5.9-9.0, preferably about 6.5, before being passed through the cation exchanger.
6. The process as claimed in one or more of the preceding claims, characterised in that the microfiltration is carried out in a microfilter having a pore diameter of 0.10-2 μm , preferably 0.4-1.5 μm .

7. The process as claimed in one or more of the preceding claims, characterised in that the saline solutions with eluted lactoperoxidase and lactoferrin, respectively, are concentrated, desalted
5 and freeze-dried.

AMENDED CLAIMS

[received by the International Bureau on 15 April 1989 (15.04.89)
original claims 1 - 7 replaced by new claims 1 - 5 (1 page)]

1. A process for extracting pure fractions of lactoperoxidase and lactoferrin from milk serum, c h a -
r a c t e r i s e d by microfiltering the milk serum,
passing it through a bed of a strong cation exchanger
5 at a high rate of flow of about 1-1.5 bed volumes/minute
for selective adsorption of lactoperoxidase and lacto-
ferrin, and then successively and selectively eluting
the lactoperoxidase with a saline solution having a
concentration of 0.10-0.4 M at a pH of about 6.5 and
10 the lactoferrin with a saline solution having a con-
centration of 0.5-2 M.

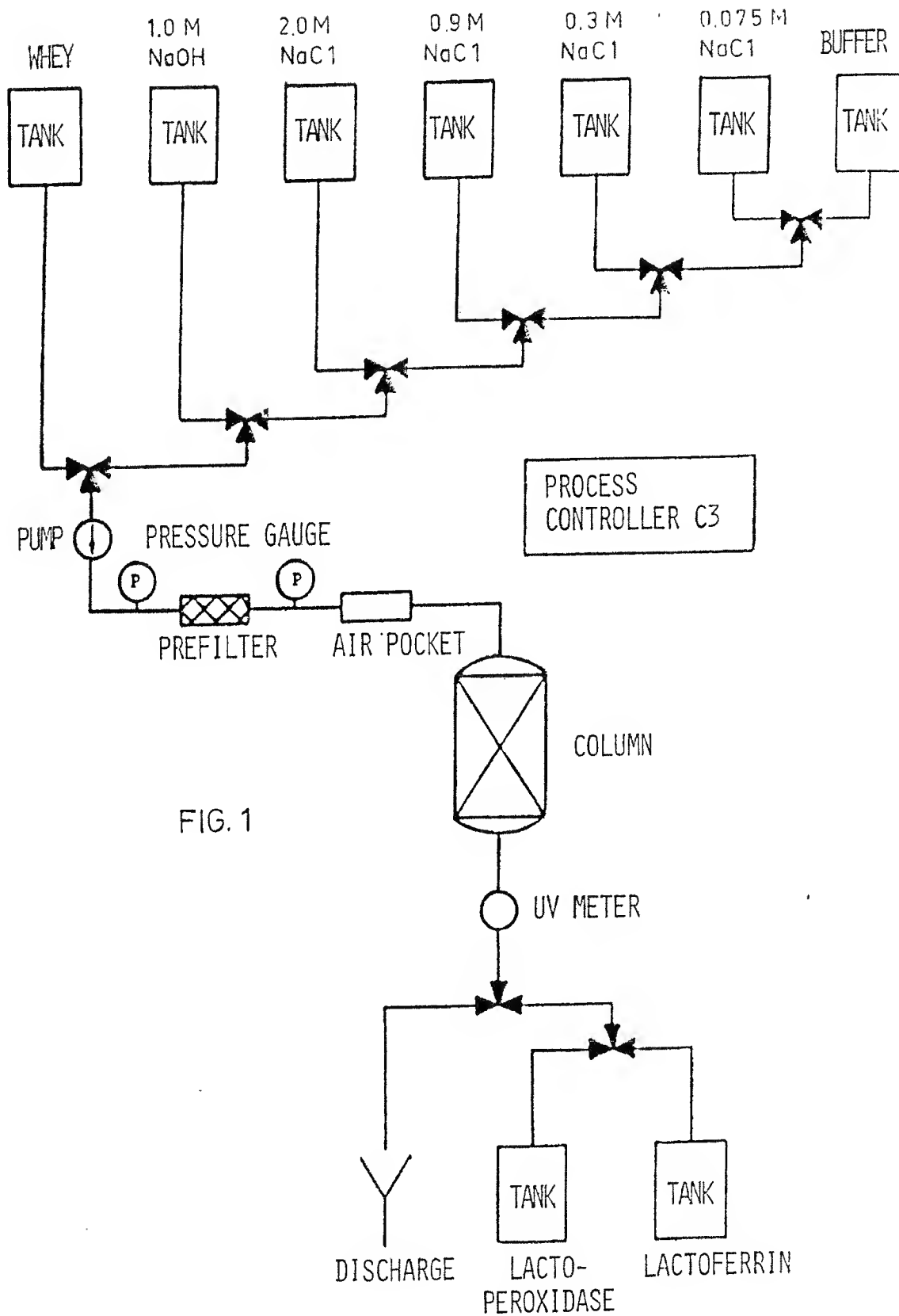
2. The process as claimed in claim 1, c h a r -
a c t e r i s e d in that before the elution of lacto-
peroxidase, the cation exchanger is eluted with a
15 saline solution having a concentration of 0.01-0.15 M,
preferably a solution of an inorganic alkali, alkaline
earth or ammonium salt.

3. The process as claimed in claim 1 or 2, c h a r -
a c t e r i s e d in that the pH of the milk serum
20 is adjusted to 5.9-9.0, preferably about 6.5, before
being passed through the cation exchanger.

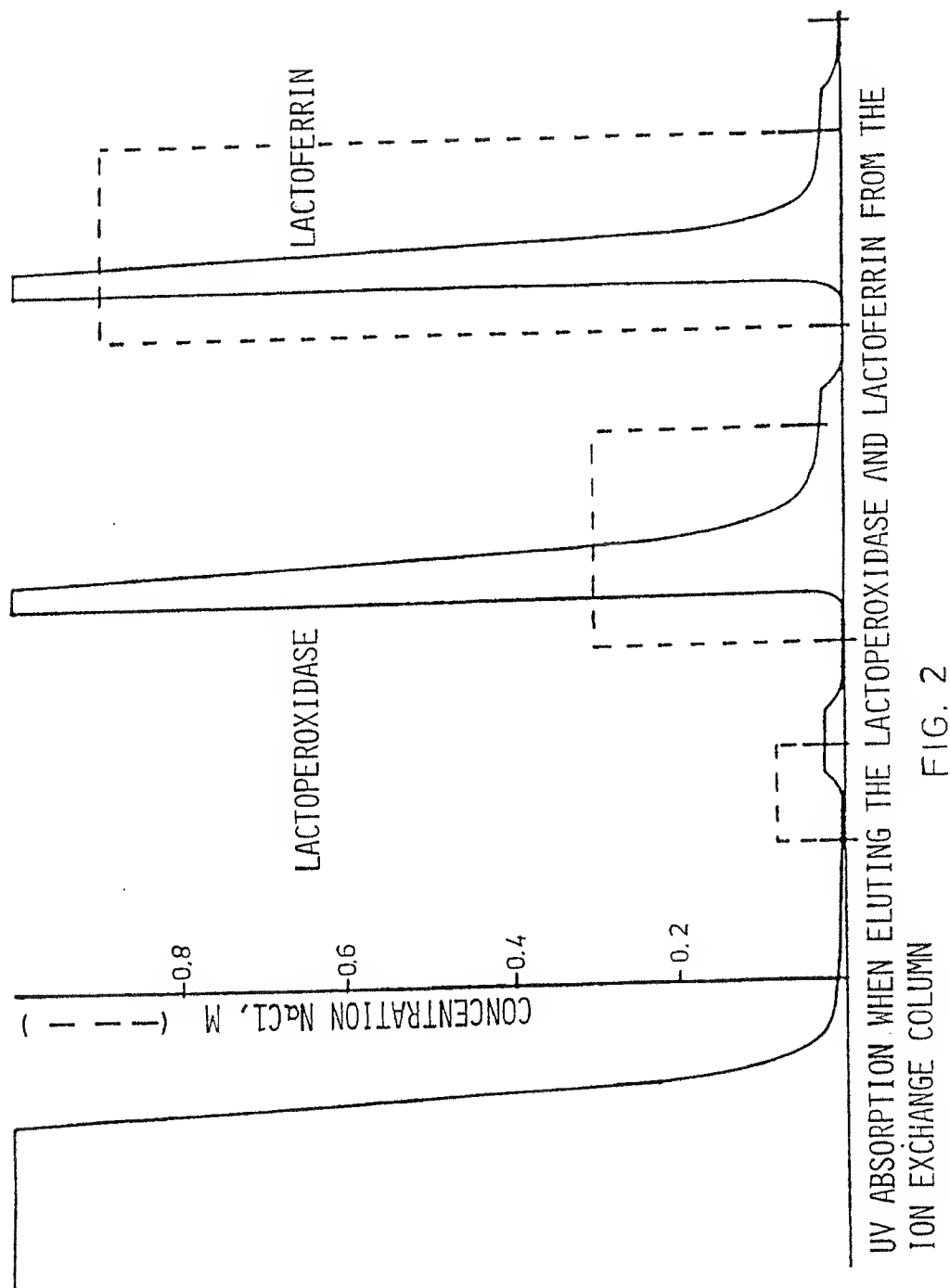
4. The process as claimed in one or more of the
preceding claims, c h a r a c t e r i s e d in that
the microfiltration is carried out in a microfilter
25 having a pore diameter of 0.10-2 μm , preferably
0.4-1.5 μm .

5. The process as claimed in one or more of the
preceding claims, c h a r a c t e r i s e d in that
the saline solutions with eluted lactoperoxidase and
30 lactoferrin, respectively, are concentrated, desalted
and freeze-dried.

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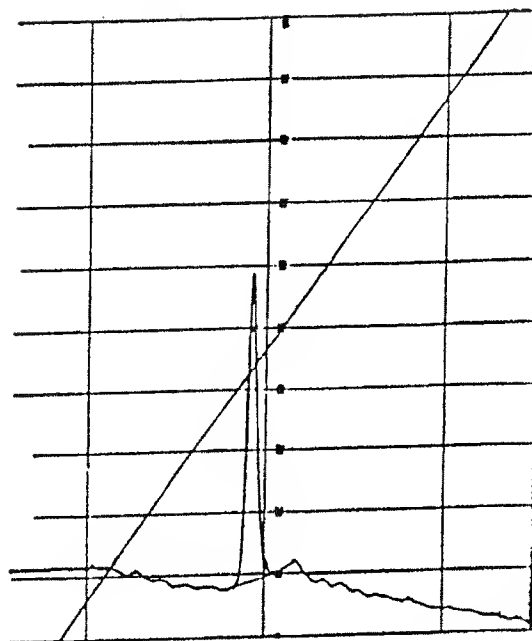


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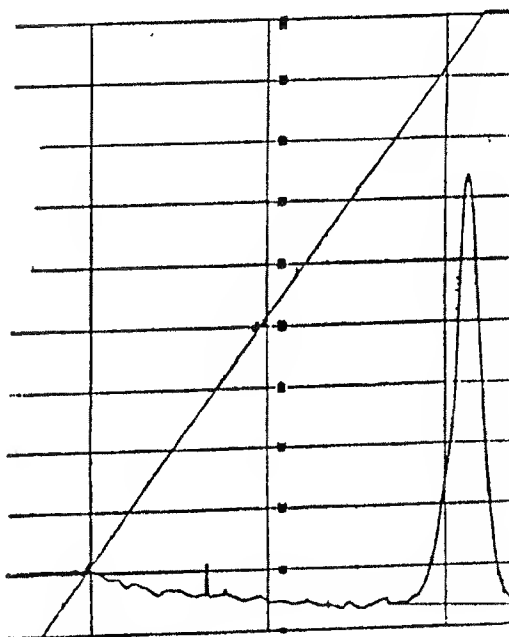


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FIG. 3



LACTOPEROXIDASE



LACTOFERRIN

CHROMATOGRAPHIC CHECK FOR THE PURITY
OF THE FRACTIONS

International Application No PCT/SE88/00643

International Application No PCT/SE88/00643

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4 436 658 (SOCIETE NATIONALE ELF AQUITAINE) 13 March 1984 & GB, 2098998 FR, 2505615 DE, 3218348 BE, 893156 NL, 8201947 SE, 8203056 JP, 58028233 CA, 1171723 CH, 650905 AU, 557353	1-7
Y	Rev. roum Biochim 21, 2, 1984, Buzila et al "The simultaneous preparation of the active components from human milk" p 81-91	1-7
Y	Chemical abstracts, Vol 105, 1986, abstract no 41350u, J Chromatogr., 1986, 358(2), 429-33(Eng)	1-7

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

II Fields searched (cont)

US C1 260:112,122,123;
435:192

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²**

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.